

the recently formed, abnormal cells. It is hoped to study the marrow folate levels in some of these patients.

5. It was shown previously<sup>28</sup> that serum vitamin B<sub>12</sub> levels rise upon administration of folate in many of these patients.

The importance of all these considerations to the clinical situation is that neither the serum vitamin B<sub>12</sub> level, nor the serum or red cell folate levels can be relied upon as indications of the deficiency that exists in our population.

This work was partly supported by the Atomic Energy Board, the South African Medical Research Council and the Chairman's Fund of the Anglo-American and De Beer's Consolidated Mines Ltd. We wish to thank our laboratory technicians, and many of our colleagues who assisted us and allowed access to their patients.

#### REFERENCES

1. Campbell, G. D. (1973): *Clinical Medicine in Africans in Southern Africa*, p. 53. Edinburgh: Churchill-Livingstone.
2. Hift, W. and Adams, E. B. (1963): *Trans. roy. Soc. trop. Med. Hyg.*, **57**, 445.
3. Keeton, G. R. (1972): *S. Afr. med. J.*, **46**, 1170.
4. Moshal, M. G. and Hift, W. (1975): *J. trop. Med. Hyg.*, **78**, 2.
5. Adams, E. B. (1957): *S. Afr. med. J.*, **31**, 633.
6. Van Rooyen, R. J., Jansen, G. J. and Meyer, B. J. (1971): *Geneeskunde*, **13**, 41.
7. Spray, G. H. (1955): *Clin. Sci.*, **14**, 661.
8. Waters, A. H. and Mollin, D. L. (1961): *J. clin. Path.*, **14**, 335.
9. Landers, J. W. and Zak, B. (1958): *Amer. J. clin. Path.*, **29**, 590.
10. Herbert, V. (1966): *J. Lab. clin. Med.*, **67**, 855.
11. Gottlieb, C., Lau, K. S., Wassermann, L. R. *et al.* (1965): *Blood*, **25**, 875.
12. Sammons, H. G., Morgan, D. B., Frazer, A. C. *et al.* (1967): *Gut*, **8**, 348.
13. Paterson, J. C. S. and Wiggins, H. S. (1954): *J. clin. Path.*, **7**, 56.
14. Schilling, R. F. (1953): *J. Lab. clin. Med.*, **42**, 860.
15. Chanarin, I. (1969): *The Megaloblastic Anaemias*, p. 317. Oxford: Blackwell Scientific Publications.
16. Nairn, R. G. (1969): *Fluorescent Protein Tracing*, 3rd ed., p. 309. Edinburgh: Blackwell Scientific Publications.
17. Fromm, F. and Hofmann, A. F. (1971): *Lancet*, **2**, 621.
18. Moshal, M. G., Lautré, G., Bader, E. *et al.* (1972): *S. Afr. med. J.*, **46**, 1892.
19. Greig, H. B. W. (1975): *Ibid.*, **49**, 1595.
20. Brandt, V., Kerrich, J. E. and Metz, J. (1963): *S. Afr. J. med. Sci.*, **28**, 125.
21. Hift, W., Moshal, M. G. and Pillay, K. (1973): *Lancet*, **1**, 570.
22. *Idem* (1973): *S. Afr. med. J.*, **47**, 915.
23. Hift, W. (1969): 'Vitamin B<sub>12</sub> and its binding', pp. 84-85, D.M. thesis, University of Oxford.
24. Gross, R. L., Reid, J. V. O., Newberne, P. M. *et al.* (1975): *Amer. J. clin. Nutr.*, **28**, 225.
25. Chanarin, I. (1969): *Op cit.*<sup>15</sup>, p. 324.
26. Hift, W. (1969): 'Vitamin B<sub>12</sub> and its binding', p. 66, D.M. thesis, University of Oxford.
27. *Idem* (1969): 'Vitamin B<sub>12</sub> and its binding', pp. 30-32, D.M. thesis, University of Oxford.
28. *Idem* (1963): *S. Afr. J. Lab. clin. Med.*, **9**, 24.

## The Adrenal Cortex in Hypercholesterolaemic Rabbits

### Histochemical and Electron Microscopical Changes

D. J. ROSSOUW, CAROL C. CHASE, F. M. ENGELBRECHT

#### SUMMARY

The adrenals of rabbits on a cholesterol-rich diet for 35 days show histopathological changes, a marked increase in weight and a lowering in the ascorbate content.

A focal increase in the neutral lipid and cholesterol content was noted mostly in the inner cortical zones; and a characteristic acid phosphatase-positive pattern in areas of infiltrating cells, and an alkaline phosphatase-positive reaction in heterophils in the infiltrated areas.

Electron microscopy confirmed that the zona glomerulosa cells were relatively normal in hypercholesterolaemic rabbits, while necrosis and fibrosis were very obvious in the inner two zones. The cellular infiltrate was shown to consist of large, granular mononuclear cells,

heterophils, eosinophils, stromal phagocytes, lymphocytes and plasma cells. The possibility that the reaction was of an immunological nature is considered.

The morphology of the adrenals of rabbits which were on a cholesterol-rich diet for 35 days and on a normal diet for 6 weeks afterwards, was indistinguishable from that of those rabbits killed after 35 days on a cholesterol-rich diet.

*S. Afr. med. J.*, **53**, 282 (1978).

Previous reports on the effects of cholesterol feeding in rabbits have indicated that certain biochemical and histopathological changes occur in the adrenal cortex, some of which seem to suggest an association with the development of the atheromatous lesions.<sup>1,2</sup> The pronounced hypertrophy of the adrenals usually preceded the phase of cholesterol deposition in the aorta,<sup>2,4</sup> and was accompanied by an infiltration of the cortex by granular leucocytes and mononuclear cells.<sup>1</sup> Adrenal enlargement, however, is neither due to increased secretion of ACTH<sup>5</sup> nor to changes in the rate of corticosterone secretion.<sup>2,6</sup>

Department of Physiology, University of Stellenbosch, Parowallei, CP

D. J. ROSSOUW, M.Sc., M.B. Ch.B.

CAROL C. CHASE, M.Sc.

F. M. ENGELBRECHT, M.Sc., D.Sc.

Date received: 25 July 1977.



Recently, research was focused on ascorbate and its significance in lipid and cholesterol metabolism and its probable relationship with atherogenesis. A relationship between the ascorbate and cholesterol metabolism was shown to exist in animals which are not capable of biosynthesizing vitamin C. In guinea-pigs significant negative correlation was shown between the tissue cholesterol concentration and the saturation of tissues with ascorbate,<sup>7</sup> while dietary supplementation with ascorbate caused an initial drop in the mean serum cholesterol concentration of captive baboons.<sup>8</sup>

Changes in certain adrenal enzymes have also been used to assess the nature of adrenal pathology in hypercholesterolaemic rabbits. Forbes *et al.*<sup>4</sup> were unable to demonstrate gross differences between cholesterol-fed and control rabbits in respect of the distribution of acid and alkaline phosphatases, succinic dehydrogenase, glucuronidase and nonspecific esterases, but reported a decrease in the ascorbate content of the adrenals. Albrecht *et al.*<sup>3</sup> described an increase in acid phosphatase activity, both in the adrenal cortices and in the accompanying atheromatous lesions in the aorta of hypercholesterolaemic rabbits. They located alkaline phosphatase activity in the adrenal medulla only, and no significant changes in its activity in response to cholesterol feeding could be demonstrated.<sup>3</sup>

In the light of these divergent results, the adrenals of normal and hypercholesterolaemic rabbits were reinvestigated by histochemical and electron microscopical techniques. The ascorbate content of the serum and adrenals was also analysed to investigate the relationship between the ascorbic acid metabolism and hypercholesterolaemia in rabbits.

## MATERIALS AND METHODS

Thirty-five New Zealand white rabbits weighing 2,0–2,5 kg were fed on a normal maintenance diet for 2 weeks before the experiment was started. Thereafter, 10 rabbits were randomly selected for the control group and kept on the maintenance diet for 35 days. The remaining 25 animals were fed on a diet supplemented with 3%

cholesterol, and 10 animals were killed after 5 days and 10 after 35 days. The remaining 5 rabbits reverted to the normal maintenance diet for a further 6 weeks, and their adrenals were processed for light and electron microscopy only. Blood samples were taken from each animal and its adrenals were carefully dissected, blotted and weighed before tissue was selected for histochemistry and microscopy. The remainder of the adrenals was processed for chemical analyses. The serum and adrenal ascorbate content was analysed according to the method described by Schaffert and Kingsley,<sup>9</sup> and serum cholesterol by the method of Engelbrecht *et al.*<sup>10</sup>

The adrenals were fixed in either phosphate-buffered neutral formalin or Bouin's fixative, and were then dehydrated, cleared and embedded in paraffin wax. Sections (5  $\mu$ m) were stained with haematoxylin and eosin, Masson's trichrome stain, periodic acid-Schiff reagent (PAS),<sup>11</sup> and aldehyde fuchsin for mast cell granules.<sup>12</sup> For histochemistry, adrenal tissue was embedded in a cube of kidney tissue and frozen on an object plate in the Cryo-cut cryostat (American Optical Co.) by use of the heat extractor. Fresh frozen sections (10  $\mu$ m) were stained with oil red O (isopropanol solution) and adjacent serial sections with haematoxylin and eosin. Similar sections were fixed in calcium formalin, rinsed and incubated in the appropriate media for the demonstration of acid and alkaline phosphatases (Gomori technique).<sup>11</sup> The adrenal glands used for the demonstration of cholesterol were fixed for 24 hours at 4°C in calcium formalin, cut into 20- $\mu$ m sections, incubated in a solution of 2,5% iron alum for 3 days at 37°C, and then stained with a premixed cholesterol reagent.<sup>13</sup>

The samples for electron microscopy were fixed in 3% phosphate-buffered glutaraldehyde, rinsed, and post-fixed in 1% osmium tetroxide in Veronal buffer (pH = 7,4). After dehydration in graded alcohols and propylene oxide the tissue was embedded in Epon 812. Semithin sections were stained with azure blue/toluidine blue, and thin sections (LKB ultramicrotome) with 3% uranyl acetate and Reynolds' lead citrate.<sup>11</sup> The thin sections were studied with a Siemens Elmiskop I electron microscope.

TABLE 1. SERUM AND ADRENAL ASCORBATE, SERUM CHOLESTEROL, BODY WEIGHT AND ADRENAL WEIGHT OF NORMAL RABBITS AND CHOLESTEROL-FED RABBITS AFTER 5 AND 35 DAYS (MEAN VALUES  $\pm$ 1 SD FOR 10 ANIMALS IN EACH GROUP)

	Serum ascorbate (mg/100 ml)	Adrenal ascorbate (mg/100 g)	Serum cholesterol (mg/100 ml)	Body weight (g)	Adrenal weight (mg)	Adrenal weight ( $\mu$ g/g body weight)
Normal rabbits	1,52 $\pm$ 0,34	229,20 $\pm$ 21,21	101,37 $\pm$ 32,67	2 395 $\pm$ 277,09	166,52 $\pm$ 23,82	70
Cholesterol-fed rabbits after 5 days	1,39 $\pm$ 0,19 NS	226,77 $\pm$ 17,08 NS	574,60 $\pm$ 58,91 †	2 482 $\pm$ 253,14 NS	155,41 $\pm$ 21,80 NS	63 NS
Cholesterol-fed rabbits after 35 days	1,29 $\pm$ 0,35 *	213,70 51,40 $\pm$ NS	826,90 $\pm$ 306,11 †	2 656 $\pm$ 194,21 NS	316,26 $\pm$ 60,29 †	119 †

NS = no statistically significant differences from control values.

\* = 0,05 < P < 0,10.

† = P < 0,05.



## RESULTS

Table I summarizes the values for the serum and adrenal ascorbate content, serum cholesterol concentration, body weight and adrenal weight of control and experimental animals. Apart from a pronounced increase in the serum cholesterol concentration, there seemed to be no significant changes after 5 days on a cholesterol-rich diet. After 35 days on the high-cholesterol diet there was a highly significant increase in both serum cholesterol levels and adrenal weight, whereas the serum ascorbate levels decreased ( $0,05 < P < 0,10$ ) without any noteworthy change in the ascorbate content of the adrenals.

Light microscopy showed no changes in adrenocortical structure after 5 days on a cholesterol-rich diet and no electron microscopy was performed. The increase in the weight of the adrenals after 35 days (Table I) confirmed our previous results, and was mainly due to a hypertrophy of the zonae fasciculata and reticularis. The accompanying



Fig. 1. The adrenal cortex of a hypercholesterolaemic rabbit, with irregular clumps of cells showing negative and positive acid phosphatase reactions lighter and darker areas respectively (Gomori technique  $\times 50$ ).

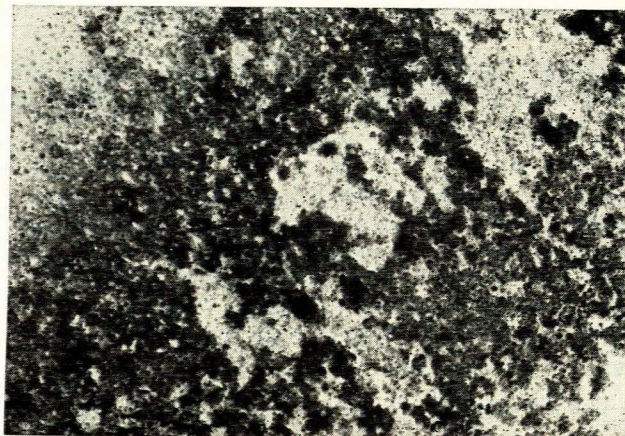


Fig. 2. Large irregular cholesterol-negative areas among the mainly cholesterol-positive cells in the adrenal cortex of a hypercholesterolaemic rabbit (frozen section; pre-mixed cholesterol reagent  $\times 50$ ).

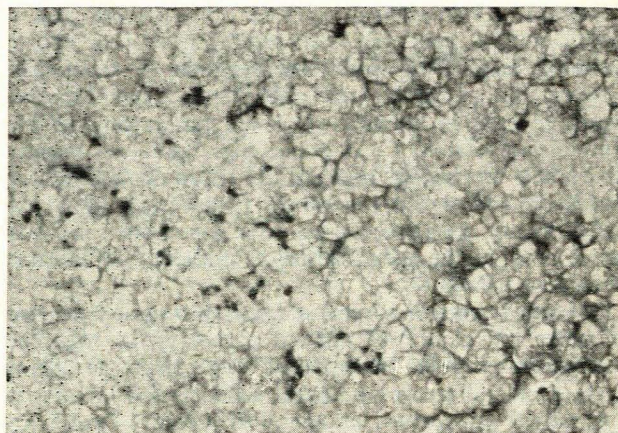


Fig. 3. Alkaline phosphatase-positive cells, presumably heterophils, appear as scattered black dots. Adrenal cortex of a cholesterol-fed rabbit (Gomori technique  $\times 130$ ).

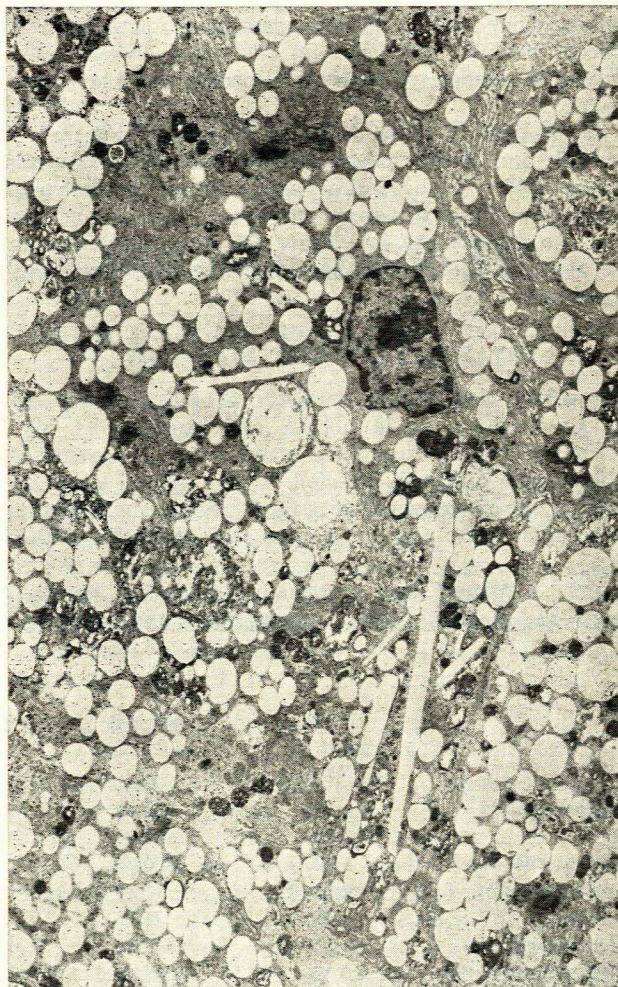


Fig. 4. Electron micrograph to show the accumulations of lipids and the degeneration of cells in the zona fasciculata of a cholesterol-fed rabbit ( $\times 3\,800$ ).



infiltration of a heterogeneous population of cells was mainly confined to the inner cortical zones and was virtually never present in the zona glomerulosa. The patchy distribution of the acid phosphatase-positive areas (Fig. 1) and similar large irregular cholesterol and oil red O-negative areas (Fig. 2), corresponded with areas infiltrated by these cells. The large mononuclear cells, both in the sinusoids and in the extravascular spaces, showed well-defined acid phosphatase-positive granules, and the randomly distributed alkaline phosphatase-positive cells (Fig. 3) most probably represented the heterophils in the cellular infiltrate.

Electron micrographs showed that the zona glomerulosa cells contained more liposomes per cell than the normal gland,<sup>9</sup> without any ultrastructural pathological changes. The zona fasciculata and reticularis, on the other hand, showed marked cellular and subcellular changes, as well as an increase in collagen fibres in the intercellular spaces. Large areas of degenerative and necrotic glandular cells were visible (Fig. 4). These cells were characterized by (a) a dense cytoplasmic matrix without any visible elements of smooth endoplasmic reticulum; (b) liposomes which lost their well-defined borders and acquired an irregular outline; (c) lipids which were visible as myelin figures (mainly phospholipids) and cholesterol clefts; and (d) lysosomes which contained semidigested lipid material and the presence of lipofuchsin bodies. Although some of these cortical cells showed a functional nucleus on electron

microscopy, many nuclei were small and pyknotic, with signs of karyolysis.

With the electron microscope it was possible to identify some of the infiltrating cells. The large mononuclear cells (Fig. 5) corresponded with the acid phosphatase-positive granular cells.

Polymorphonuclear heterophils (Fig. 6) and eosinophils were identified by their characteristic cytoplasmic granules. A marked proliferation of fibroblasts, macrophages, lymphocytes and plasma cells was observed, mainly in the spaces between the glandular cells (Fig. 7). Fibroblasts, with dilated granular endoplasmic reticulum and a well-developed Golgi area, were embedded in collagen fibres. In these areas a very characteristic association between macrophages and lymphocytes, and between lymphocytes and plasma cells was a consistent finding.

Adrenal histopathology of rabbits which were fed a normal diet for 6 weeks after being fed a cholesterol-rich diet for 35 days, was indistinguishable from that of rabbits killed at the end of 35 days on a cholesterol-rich diet.



Fig. 5. Typical mononuclear granular cell in a cortical sinusoid ( $\times 6700$ ).

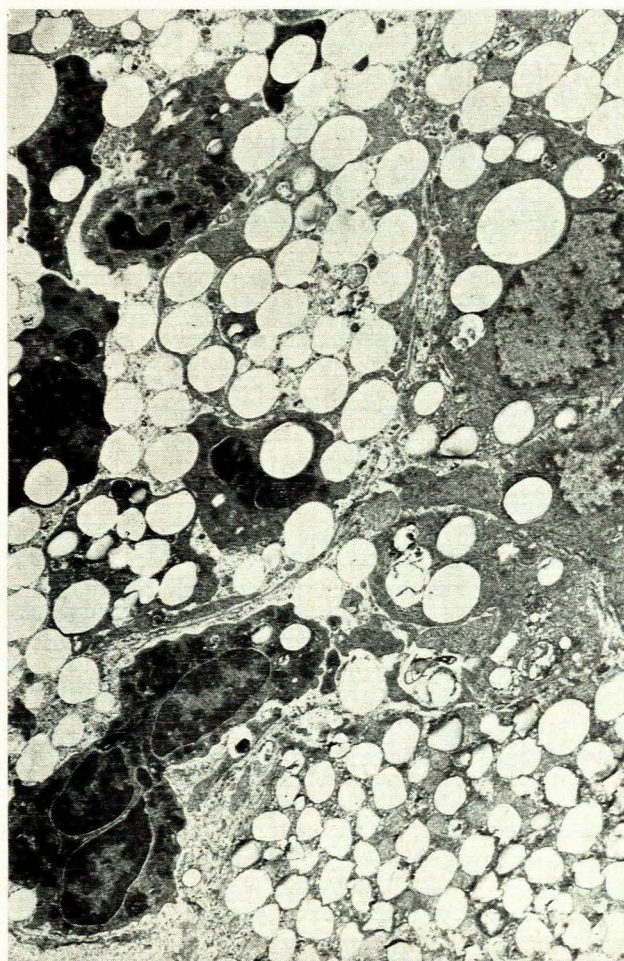


Fig. 6. Polymorphonuclear heterophils infiltrating the adrenal cortex of a hypercholesterolaemic rabbit. The heterophil in the bottom left-hand corner shows a tri-lobular nucleus and is abnormally electron-dense ( $\times 3200$ ).



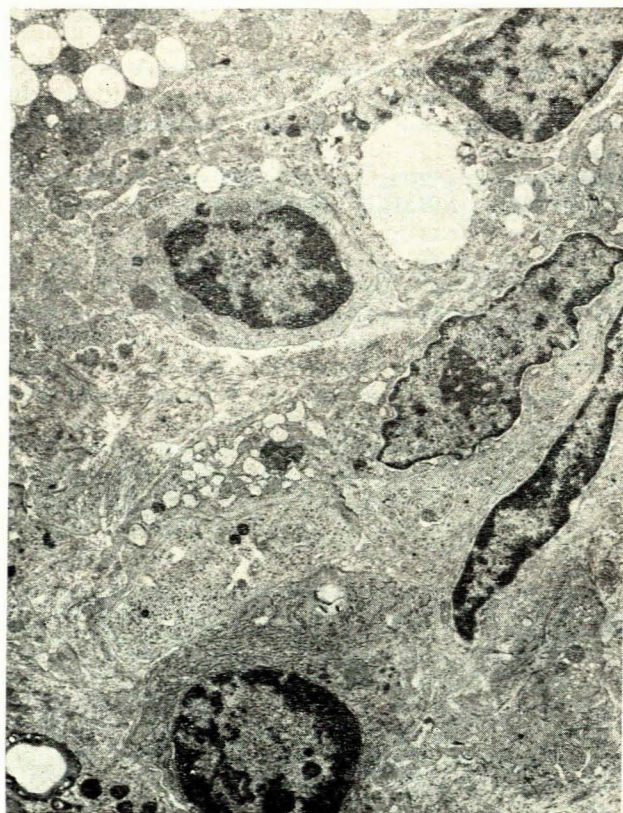


Fig. 7. Electron micrograph to show the non-glandular cells and collagen fibres in the space between glandular cells of the adrenal cortex of a cholesterol-fed rabbit. Note the close association of the lymphocyte and macrophage (top centre); the dilated cisternae of granular endoplasmic reticulum in the fibroblast (centre); and the lymphocyte and plasma cell (bottom centre) ( $\times 6000$ ).

## DISCUSSION

Histochemical tests have shown that cholesterol feeding depleted the ascorbate content of the zona fasciculata.<sup>4</sup> However, we were unable to demonstrate any significant changes in the ascorbate content per unit weight (Table I), despite a highly significant increase in the adrenal weight. We therefore felt that the adrenal pathology in hypercholesterolaemic rabbits is probably related to factors other than changes in ascorbate metabolism, although the latter may be involved in some way.

Our results showed no alkaline phosphatase reaction in the normal adrenal medulla.<sup>9</sup> The randomly distributed alkaline phosphatase-positive cells in the inner cortical zones of cholesterol-fed animals helped us to identify the polymorphonuclear heterophils among the infiltrating cells (Fig. 3). Acid phosphatase located in the adrenal cortex is closely associated with lysosomes and the accumulation of lipids in adrenocortical cells. The patchy distribution of acid phosphatase-positive areas in the adrenals of cholesterol-fed rabbits (Fig. 1) corresponded with foci of infiltrating cells and areas of cortical necrosis. Albrecht *et al.*<sup>3</sup> also found an increase in acid phosphatase activity which was related to the duration of cholesterol feeding,

and it persisted after the cholesterol diet had been withdrawn and replaced by the normal diet for almost 2-3 months.

No literature on the ultrastructural pathology of the adrenal cortex in hypercholesterolaemic animals could be found. Our electron micrographs showed the zona glomerulosa to be relatively normal after rabbits had been fed a 3% cholesterol diet for 35 days. It appeared, therefore, that the glomerulosa cells which are not under ACTH regulation and which synthesize mainly mineralocorticoids behave quite differently from the glucocorticoid-secreting cells when rabbits become hypercholesterolaemic.

The fine structure of the degenerative and fibrotic cortical areas indicated that the accumulation of lipids and an almost simultaneous increase in the intercellular collagen fibres are the first ultrastructural changes. It seems likely that the inability of the lysosomes to digest the large amounts of accumulated lipids in the glandular cells or the release of lysosomal enzymes from the infiltrating phagocytic cells may play a role in the initiation of adrenocortical degeneration and fibrosis. Finally, it is possible that the very characteristic and consistent presence of macrophages, lymphocytes and plasma cells (Fig. 7) may indicate an immunological basis for the adrenocortical pathology. Whether the hypergammaglobulinaemia found in hypercholesterolaemic rabbits<sup>15</sup> should be seen as an additional sign of such an immunological reaction is not clear at the moment and needs to be investigated further.

Our studies indicated that there were no signs of reversion of the adrenocortical lesions 6 weeks after the animals had been returned to a normal diet. Pula *et al.*<sup>14</sup> recently found that if rabbits were fed on a cholesterol-supplemented diet for 2 months, the normal structure of the cortex had not been restored even 6 months after the resumption of the normal diet. It therefore seems likely that the adrenal lesions induced by hypercholesterolaemia are not dependent on the sustained presence of the primary stimulus. In view of these known alterations in adrenocortical structure and function during hypercholesterolaemia, as well as the influence exerted by the adrenals on metabolic and immunological processes, there appears to be some justification for assuming that the adrenal glands play a role in the development of atherosclerosis.

## REFERENCES

1. Rossouw, D. J. and Engelbrecht, F. M. (1973): *S. Afr. med. J.*, **47**, 1460.
2. Albrecht, W. and Schuler, W. (1965): *J. Atheroscler. Res.*, **5**, 353.
3. Albrecht, W., Kahnt, F. W., Neher, R. *et al.* (1965): *Ibid.*, **5**, 365.
4. Forbes, W., Munro, H. N. and Steele, M. H. (1962): *J. Physiol.*, **164**, 31P.
5. Forbes, W., Steele, M. H. and Munro, H. N. (1964): *Brit. J. Nutr.*, **18**, 55.
6. Morris, M. D., Fisher, D. A. and Krum, A. A. (1966): *J. Atheroscler. Res.*, **6**, 283.
7. Ginter, E., Cerven, J., Nemec, R. *et al.* (1971): *Amer. J. clin. Nutr.*, **24**, 1238.
8. De Klerk, W. A., Kotzé, J. P., Weight, M. J. *et al.* (1973): *S. Afr. med. J.*, **47**, 1503.
9. Schaffert, R. R. and Kingsley, G. R. (1955): *J. biol. Chem.*, **212**, 59.
10. Engelbrecht, F. M., Mori, F. and Anderson, J. T. (1974): *S. Afr. med. J.*, **48**, 250.
11. Chase, Carol C. (1976): M.Sc. thesis, University of Stellenbosch, Stellenbosch, CP.
12. Lillie, R. D. (1965): In *Histopathologic Technic and Practical Histochemistry*, 3rd ed., p. 556. New York: McGraw-Hill.
13. Rossouw, D. J., Chase, C. C., Raath, I. *et al.* (1976): *Stain Technology*, **51**, 143.
14. Pula, G., Toti, P. and Weber, G. (1975): *Boll. Soc. ital. Biol. sper.*, **51**, 97.
15. Rossouw, D. J. and Engelbrecht, F. M. (1973): *S. Afr. med. J.*, **47**, 2154.